SCREENING OF ANTIMICROBIAL ACTIVITIES BY AQUATIC HYPHOMYCETES CULTIVATED ON VARIOUS NUTRIENT SOURCES.

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ABSTRACT. — Twenty-six strains belonging to 19 species of aquatic hyphomycetes isolated from freshwater streams in Spain were tested for antimicrobial activities after cultivation on 24 different solid media. Fifteen of these cultures produced antibacterial activities and 8 produced antifungal activities in at least one medium. The most succesful media were those with a low nitrogen/carbon ratio. The ecological and biotechnological implications of these results are discussed.

KEY WORDS : antibacterials, antifungals, ingoldian fungi, production media.

RÉSUMÉ. — Trente six souches de 19 espèces d'hyphomycètes aquatiques, isolées de rivières d'Espagne, ont été testées pour leur activité antimicrobienne sur 24 milieux de culture différents. Quinze de ces souches présentent une activité antimicrobienne et 8 une activité antifongique, sur au moins un milieu. Les meilleurs milieux sont ceux présentant le plus faible rapport azote/carbone. Les implications écologiques et biotechnologiques de ces résultats sont discutées.

MOTS CLEFS : antibactérien, antifongique, ingoldien, milieu

INTRODUCTION

The significant nutritional and environmental differences between aquatic and terrestrial habitats could select for the development of secondary metabolic pathways in aquatic fungi, that differ from those of other fungi. During this last decade, surveys of different aquatic habitats for novel secondary metabolite producers, have resulted in the identification of several species of fungi that synthesize previously undiscovered biologically active compounds. Some examples of the organisms isolated from different aquatic sources are *Microascus longirostris* Zukal (Yu *et al.*, 1996) directly isolated from water;

Source : MNHN, Paris

marine animal parasites or commensals like *Phoma* sp. (Sugano *et al.*, 1991); fungi isolated from seaweeds like *Leptosphaeria* sp. (Takahashi *et al.*, 1995a, 1995b), *Asteromyces cruciatus* F. et F. Moreau ex Hennebert (Shin & Fenical, 1987); or endophytes like *Leptosphaeria obiones* (Crouan & Crouan) Saccoro (Poch & Gloer, 1989b) and *Helicascus kanaloanus* Kohlmeyer (Poch & Gloer, 1989a). Most of these organisms were isolated from marine samples, although only some could be considered true marine fungi.

However, other aquatic ecosystems have been less reported or investigated as sources for potential producers of biologically active natural products. This is the case for freshwater habitats and aquatic hyphomycetes, ■ taxonomically and phylogenetically heterogeneous group of fungi that constitute the dominant group of organisms colonizing deciduous leaves and submerged woods in freshwater courses. It has been reported that this group of organisms is able to produce substances with biotechnological applications, such as enzymes (xylanases, pectinases, proteases, etc.) (Suberkropp, 1992). Nevertheless, although some ecological evidence suggests that members of this group produce diffusible inhibitory substances (Shearer & Zare-Maivan, 1988; Fisher & Anson, 1983), only two active compounds have been fully characterized up to date : kirschsteinin, a cytotoxic compound isolated from *Kirschteiniothelia elaterascus* Shearer (Poch & Gloer, 1992), and anguillosporal, an antibacterial and antifungal compound isolated from *Anguillospora longissima* (Sacc. & Syd) Ingold (Harrigan *et al.*, 1995).

Also, the ecological characteristics shared by these aquatic hyphomycetes could make them share their metabolic response to different growth conditions, in spite of their phylogenetic heterogeneity. The goal of the present work is to determine the influence of nutrients on the production of antimicrobial activities by these fungi. The information obtained from these experiments is expected to be applicable to the design of production media to be included in ■ screening of natural products from aquatic fungi. With this purpose, we selected for this study 26 fungal strains, belonging to 19 species of aquatic hyphomycetes isolated from freshwater streams in several locations in Spain.

To study the influence of nutrients on the production of diffusible antimicrobial compounds, the fungi were grown on 24 different agar media formulated with complex sources commonly used in fungal fermentations. The choice of using these complex sources for this experiment, instead of chemically defined media, is because they usually produce a better yield of secondary metabolites. The production of antimicrobial activities against a representative bacterium (*Bacillus subtilis*), and a representative fungus (*Aspergillus fumigatus*) was evaluated after two and four weeks of incubation.

MATERIALS AND METHODS

Fungal strains :

The aquatic hyphomycetes used for this study (Table 1) were monosporic isolates collected from different freshwater streams in Spain, stored at CIBE (Centro de Investigación Básica España) culture collection. The selection of the cultures to be studied was performed to achieve both a large number of species and a high range of taxonomic variability, as it is shown by the identified teleomorphs.

Source : MNHN, Paris

Table 1. Cultures studied and its teleomorph.

Strain code	Species	Collection place	Teleomorph
FP 189	*Alatospora acuminata* Ingold	Madrid	Unknown
FP 400	*Alatospora acuminata* Ingold	Asturias	Unknown
FP 48	*Anguillospora crassa* Ingold	Avila	*Mollisia uda* (Pers.: Fr.) Gill. (Leotiales)
FP 154	*Anguillospora crassa* Ingold	Segovia	*Mollisia uda* (Pers.: Fr.) Gill. (Leotiales)
FP 216	*Anguillospora crassa* Ingold	Avila	*Mollisia uda* (Pers.: Fr.) Gill. (Leotiales)
ED 61	*Anguillospora* sp.	Mallorca	
FP 129	*Articulospora tetracladia* Ingold	Segovia	*Ombrophila tetracladia* (Abdullah *et al.*) Baral & Krieglsteiner (Leotiales)
ED 67	*Dendrospora tenella* Descals & Webster	Avila	Unknown
ED 64	*Fillosporella* sp.	Leon	Unknown
FP 168	*Flagellospora* sp.	Segovia	Unknown
FP 225	*Gyoerffyella* sp.	Madrid	Unknown
FP 204	*Heliscella stellata* (Ingold & Cox) Marvanová	Avila	Unknown
FP 138	*Heliscus lugdunensis* Saccardo. & Therry	Segovia	*Nectria lugdunensis* Webster (Hypocreales)
FP 373	*Heliscus lugdunensis* Saccardo. & Therry	Asturias	*Nectria lugdunensis* Webster (Hypocreales)
FP 273	*Margaritispora aquatica* Ingold	Avila	Unknown
FP 159	*Tetrachaetum elegans* Ingold	Segovia	Unknown
FP 199	*Tetracladium setigerum* (Grove) Ingold	Avila	Unknown
ED 21	*Tricladium curvisporum* Descals	Leon	Unknown
ED 45	*Tricladium splendens* Ingold	Leon	*Hymenoscyphus splendens* Abdullah *et al.* (Leotiales)
FP 27	*Tricladium splendens* Ingold	Avila	*Hymenoscyphus splendens* Abdullah *et al.* (Leotiales)
ED 31	*Tumularia aquatica* (Ingold) Descals & Marvanová	Leon	*Massarina aquatica* Webster (Dothideales)
FP 148	*Tumularia aquatica* (Ingold) Descals & Marvanová	Segovia	*Massarina aquatica* Webster (Dothideales)
ED 60	*Tumularia tuberculata* (Gonczol) Descals & Marvanová	Mallorca	Unknown
FP 2	*Varicosporium elodeae* Kegel	Avila	Unknown
FP 164	*Varicosporium elodeae* Kegel	Segovia	Unknown
FP 384	Hyphomycete undetermined	Asturias	Unknown

Source : MNHN, Paris

Cultural conditions :

Plugs of actively growing fungal mycelia, grown on potato dextrose agar (Difco) (0.5 mm^2), were placed on the surface of 9 cm diameter Petri dishes containing 20 ml of medium composed by 1% nutrient and 2% agar. Duplicates of these plates were incubated for 2 and 4 weeks respectively at 22°C. The complex nutrient sources tested were : yellow corn meal (Quaker), corn steep liquor (Roquette), pharmamedia (Traders Protein), malt extract, casein hydrolysate, peptonized milk (Oxoid), molasses (Riverton), soy meal, carboxymethylcellulose (Sigma), tomato paste (HUNBS), yeast extract, tryptone, bacto-peptone, beef extract, brain heart infusion, casamino acids, skim milk, potato dextrose broth (Difco), amicase, ardamine, N-Z-Amine E, Hy-Soy (Sheffield), soluble starch (USB) and oat meal (Santivery).

Antimicrobial activity assays :

Antibacterial assay. The antibacterial assay Petri dishes were prepared with 10 ml of melted nutrient agar (Difco) inoculated with *Bacillus subtilis* (0.5 ml of a spore suspension (Difco) per 1 liter of melted nutrient agar (Difco) cooled at 45°C)

Antifungal assay : The antifungal Petri dishes were prepared with 10 ml of melted YNB-D medium (dextrose 1%, yeast nitrogen base (Difco) 0.7% and agar 1.5%), cooled at 45°C and inoculated with a titrated spore suspension of *Aspergillus fumigatus* Fres. ATCC 13073. The final concentration of spores was 5×10^5 spores/liter.

To perform the antimicrobial tests, 8 mm diameter agar plugs were cut from plates with hyphomycetes after 2 and 4 weeks of incubation, and placed over the surface of the assay plates, and were incubated overnight at 37°C. The diameter of the inhibition zone around each agar plugs was recorded.

RESULTS

Fifteen strains, belonging to 11 fungal species, produced antibacterial activity under at least, one cultivation condition (Table 2). The production of antifungal activities was not as frequent, being observed in 8 strains belonging to 6 fungal species (Table 3). Both antibacterial and antifungal activities were detected with 5 strains :*Dendrospora tenella*, *Gyoerffyella* sp., *Tumularia aquatica* ED 31 and FP 148 and *Varicosporium elodeae* FP 2. In one case, *Gyoerffyella* sp., the inhibition of both target strains was detected under the same cultural conditions, suggesting the presence of a single activity with both antibacterial and antifungal properties, or a common stimulation of the production of several agents. The tested strains of *A. acuminata*, *A. tetracladia*, *Fillosporella* sp., *H. stellata*, *T. curvisporum* and *T. splendens*, did not produce antimicrobial activities in the conditions studied.

The data suggest that both types of antimicrobial activities are released at different incubation times; 77% of the 63 antifungal activities were detected at 2 weeks, being 35 activities detected both at 2 and 4 weeks. However, only 48% of the 82 antibacterial activities detected were shown at 2 weeks, being 30 of them also observed at 4 weeks. Assuming that the detection of these antimicrobial activities at 2 time periods is due to the

Source : MNHN, Paris

Table 2. Presence of inhibition zones against *B. subtilis* produced by hyphomycete strains

Source	*Dendrospora tenella* ED 67	*Flagellospora* sp. FP 168	*Gyoerffyella* sp. FP 225	*Varicosporium elodeae* FP 2	*Anguillospora crassa* FP 216	*Anguillospora* sp. ED 61	*Tetrachaetum elegans* FP 159	*Tetracladium setigerum* FP 199	*Varicosporium elodeae* FP 164
soy meal	-	-	-	-	-	-	-	-	-
oat meal	-	-	-	-	-	-	-	-	-
corn meal	-	-	-	-	-	-	-	-	-
tomato paste	-	-	-	-	-	-	-	-	-
potato dextrose	+-	-	-	+-	-	-	-	-	-
malt extract	++	-+	++	-+	-	-	-	-	-
molasses	++	-+	++	++	-	-	-	-	-
soluble starch	++	-+	++	-+	-	-	-	-	-
carboxymethylcellulose	-	-	-	-	-	-	-	-	-
pharmamedia	-	-	-	-	-	-	-	-	-+
corn steep liquor	-	-	-	-	-	-	-+	-+	-+
Hy-soy	-	-	-	-	-	-	-	-	-
ardamine	-	-	-	-	-	-	-	-	-
yeast extract	-	-	-	-	-	-	-	-	-
brain heart infusion	-	-	-	-	-	++	-	-	-
bactopeptone	-	-	-	-	-	-	-	-	-
beef extract	-	-	-	-	-+	-	-	-	-
skim milk	-	-	-	-	-	-	-	-	-
peptonized milk	-	-	-	-	-	-	-	-	-
N-Z-amine E	-	-	-	-	-	++	-	-	-
casein hydrolysate	-	-	-	-	-	-	-	-	-
amicase	-	-	-	-	-	-	-	-	-
casamino acids	-	-	-	-	-	-	-	-	-
tryptone	-	-	-	-	-	++	-	-	-

+-. Activity detected only at 2 wk.
-+. Activity detected only at 4 wk.
++. Activity detected at 2 and 4 wk.
-. No activity

Source : MNHN, Paris

Table 2. Continued

Source	*Anguillospora crassa* FP 48	*Anguillospora crassa* FP 154	*Margaritispora aquatica* FP 273	*Tumularia aquatica* ED 31	*Tumularia aquatica* FP 148	*Tumularia tuberculata* ED 60
soy meal	-	-	-	++	++	-+
oat meal	-	-	-	-+	-+	++
corn meal	-	-	-	++	-	++
tomato paste	-	+-	-	+-	-	++
potato dextrose	+-	++	+-	++	++	-+
malt extract	++	++	-	-	-	-
molasses	++	++	-	-	-	-
soluble starch	-+	-+	-	-	-	-
carboxymethylcellulose	-	-	-	++	-	-+
pharmamedia	-	-	-	-	-	-+
corn steep liquor	-	-	-	-+	-	-+
Hy-soy	-	-	-	-	-	-
ardamine	-	-+	-	++	-	-
yeast extract	-+	-+	-	++	-	-+
brain heart infusion	-	-	-	-	-+	-
bactopeptone	-	-+	-	-+	-	-+
beef extract	-	-	-	++	-+	-+
skim milk	-	-	+-	-+	-+	-+
peptonized milk	-	-+	-	-	-	-+
N-Z-amine E	-	-	-	-+	-	-
casein hydrolysate	-	-	-	++	-	+-
amicase	-	-+	-	+-	-	-+
casamino acids	-+	-+	-	++	-	++
tryptone	-	-+	-	-+	-	-

+-. Activity detected only at 2 wk.
-+. Activity detected only at 4 wk.
++. Activity detected at 2 and 4 wk.
-. No activity

Source : MNHN, Paris

Table 3. Presence of inhibition zones against *A. fumigatus* produced by hyphomycete strains.

Source	*Dendrospora tenella* ED 67	*Gyoerffyella* sp. FP 225	*Varicosporium elodeae* FP 2	*Heliscus lugdunensis* FP 138	*Heliscus lugdunensis* FP 373	Hyphomycete 1 FP 384	*Tumularia aquatica* ED 31	*Tumularia aquatica* FP 148
soy meal	-	-	-	-	-	-	++	++
oat meal	-	-	-	-	-	-	+-	-
corn meal	-	-	-	-	-	-	+-	-
tomato paste	-	-	-	-+	-	-	++	++
potato dextrose	-	-	+-	++	++	-	-	-
malt extract	++	++	++		-	-	-	-+
molasses	++	++	++	-+	-	-	+-	-+
soluble starch	-	-+	-	-	-	-	-	-
carboxymethylcellulose	-	-	-	+-	-	-	-	-
pharmamedia	-	-	-	-	-	-	-	-
corn steep liquor	-	-	-	-	-	-	-	-
Hy-soy	-	-	-	+-	-+	-	++	++
ardamine	-	-	-	++	++	-	-	-
yeast extract	-	-	-	++	++	-	-	-+
brain heart infusion	-	-	-	++	+-	+-	++	-
bactopeptone	-	-	-	+-	-	-	-	-
beef extract	-	-	-	-	-+	-	-+	-+
skim milk	-	-	-	++	++	-	-+	-+
peptonized milk	-	-	-	++	+-	-	++	-+
N-Z-amine E	-	-	-	++	-	-	++	++
casein hydrolysate	-	-	-	-+	-	-	++	+-
amicase	-	-	-	++	+-	++	++	+-
casamino acids	-	-	-	-	-	-	++	++
tryptone	-	-	-	++	-	-	++	++

+-. Activity detected only at 2 wk.
-+. Activity detected only at 4 wk.
++. Activity detected at 2 and 4 wk.
-. No activity

Source : MNHN, Paris

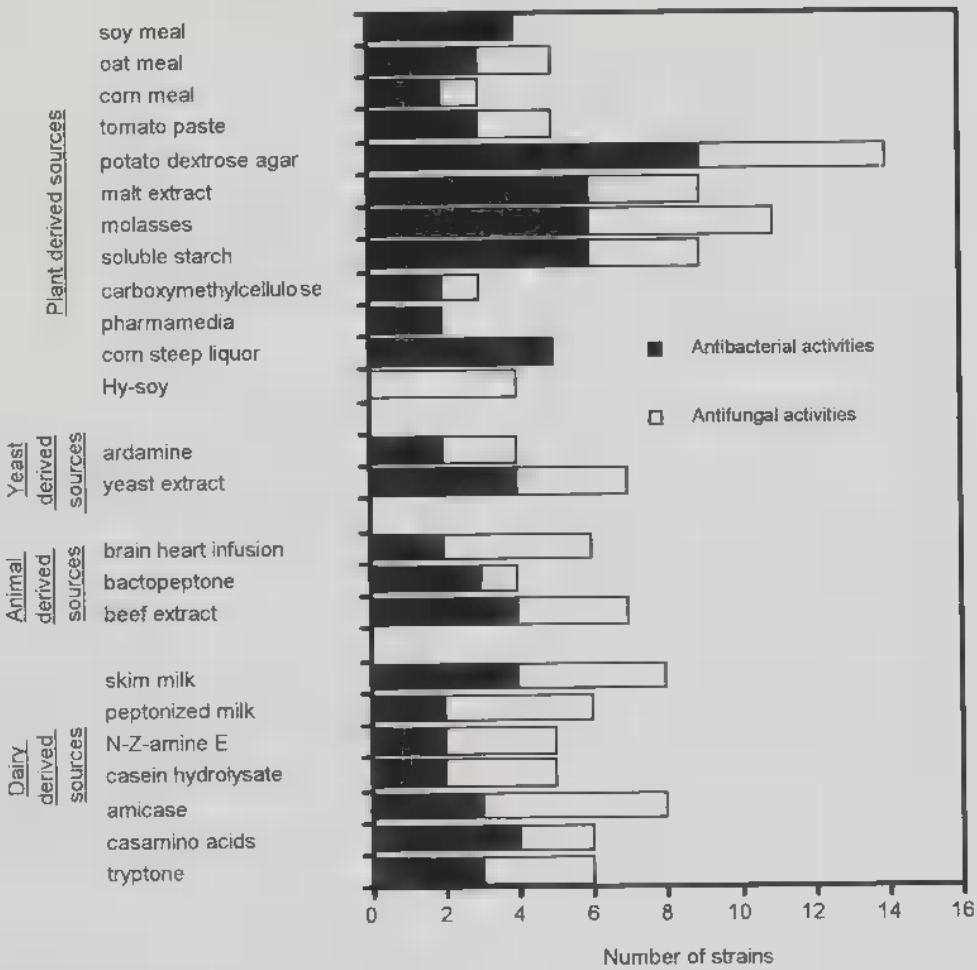

Figure 1. — Number of hyphomycete strains having antimicrobial activities when cultured in a single nutrient.

action of the same active agent(s), this result could suggest either that the antimicrobial compounds produced were relatively stable, or that they were being continuously synthesized along the whole course of the incubation.

Although the production of antimicrobial activities in all the hyphomycetes tested is not induced by a given complex source, some of them, such as potato dextrose, molasses, malt extract and soluble starch, were especially useful (Fig. 1). These media share a low nitrogen/carbon ratio, characteristic of traditional fungal media. Nevertheless, other nutrient sources with a higher nitrogen /carbon balance such as beef extract or skim milk also supported the production of biological activities in a considerable number of strains.

Source : MNHN, Paris

The conditions and ability for the production of antimicrobial activities varied among strains of the same species. For example, in the three strains of *A. crassa*, the number of sources that stimulate the production of the antibacterial activity in FP 48, FP 154 and FP 216 were 6, 12 and 1, respectively.

The production of antimicrobial agents in response to the different media by the aquatic hyphomycetes studied, was very variable. For instance, *T. setigerum* and *T. elegans* produced antibacterial activities only under one condition. *Dendrospora tenella*, *Flagellospora* sp., *Gyoerffyella* sp. and *V. elodeae* FP 2 produced these activities in carbohydrate enriched media such as malt extract, molasses and soluble starch. Finally, some fungi such as *H. lugdunensis* or species of the genus *Tumularia* produced antimicrobial activity when grown in several sources with no related ingredients.

DISCUSSION

The production of diffusible antibiotic substances is a widely distributed characteristic among fungi. Although it is difficult to infer the role of these compounds in natural environments or if they occur at all, they could provide an ecological advantage in the immediate micro habitats of the producer organisms by defending its nutritional or positional resources from other competitors. Theoretically, this statement may be less applicable to aquatic environments, because of the ecological ineffectiveness of a compound that is constantly removed by water. Therefore, although aquatic hyphomycetes most likely persist in twigs and leaves that are exposed to terrestrial environments, the ecological preeminence of a fungus able to produce antibiotics in aquatic systems is not so obvious. In any case, the ability of several aquatic hyphomycetes to produce these compounds has been shown previously (Shearer & Zare-Maivan, 1988; Fisher & Anson, 1983), and has been also extensively observed in this work.

The substrates inhabited by this group of fungi, deciduous leaves and submerged woods, due to their lignin enriched composition are hypothetically more susceptible to fungal rather than to bacterial colonization. For that reason, as the major competitors of these organisms in nature should be other fungi, aquatic hyphomycetes would be expected to produce a fungal rather than a bacterial inhibition. However, in the survey presented in this work, from 19 fungal species tested, 11 appear to produce bacterial inhibition and only 6 produce antifungal compounds. Although the extrapolation of these observations to the real interactions of these fungi in natural environments could be undoubtfully questioned, these data may suggest the importance of bacterial-fungal interactions in the colonized wood habitat. The release of an antibacterial agent could limit the consumption by bacteria of the sugars and other nutrients liberated by the extracellular enzymatic machinery of the fungi (cellulases, xylanases, pectinases, etc.).

Antifungal agents not only interfere with the growth of other hyphomycetes but also affect the viability of yeasts. Yeasts, like bacteria, are commonly found in freshwater courses, have high growth rates, and could behave as opportunistic commensals of the substances liberated by them. It has been reported that wood blocks colonized by *Tumularia aquatica* (syn. *Massarina aquatica*) were able to inhibit the growth of the yeast *Sporobolomyces roseus* (Fisher & Anson, 1983). The 2 different strains of *Tumularia aquatica* studied in this work also produced an antifungal activity that caused the inhibition of *Candida albicans* (data not shown), together with an antibacterial agent, whose production did not always coincide with the detection of the antifungal activity.

Source : MNHN, Paris

Heliscus lugdunensis, a species from submerged twigs and leaves, produced an antifungal activity. Shearer & Zare-Maivan (1988), studying the *in vitro* interactions among different fungi from freshwater habitats, have suggested that this species might not be able to inhibit growth of invading species or to defend captured resources. We observed antifungal activities in the two *H. lugdunensis* strains studied, FP 138 and FP 373. These data do not agree with the above mentioned suggestion, perhaps because their competition experiments were performed on corn meal agar, medium in which we were also unable to detect the antifungal activity produced by this organism (Table 3). Also, it has to be considered that the production of the antifungal agent shows strain-to-strain variations, as evidenced by the fact that from the sixteen cases in which an antifungal activity appeared, only 8 were common between both isolates. The potentially different susceptibility of *A. fumigatus* and the aquatic hyphomycetes studied by Shearer & Zare-Maivan to the antifungal compound, could also contribute to explain the different results.

As mentioned above, 5 strains inhibited both target organisms. However, the number of these antimicrobial activities could be higher, because the detection of an antimicrobial activity is related to the concentration tested and, also, to the susceptibility of the target strain to the active agent(s). As an example, anguillosporal from *Anguillospora longissima* (Sacc. & Syd.) Ingold (Harrigan *et al.*, 1995) is able to inhibit both bacteria and fungi, with MIC values of 4 µg/ml against *Staphylococcus aureus* and 58 µg/ml against *Candida albicans*. The ecological impact of a broad spectrum antibiotics in natural conditions is unknown. However, in terms of metabolic economy, it may be considered that the synthesis of a general inhibitor could be less expensive than the production of several specific compounds.

This work has also shown the heterogenous behavior of this group of fungi with regards to the conditions required for antibiotic production. Based on this aspect, the hyphomycetes studied could be divided in four groups : i) fungi producing the antimicrobial activity selectively in media with a very low nitrogen/carbon balance (malt extract, molasses, soluble starch or potato dextrose), such as *D. tenella*, *Gyoerffyella* sp., *V. elodeae* FP 2, and *Flagellospora* sp.; ii) fungi that produce the antimicrobial activity only in media with ■ high nitrogen/carbon balance, (*A. crassa* FP 216, ED 61 and Hyphomycete 1); iii) fungi whose antimicrobial activity production is less dependent of the nitrogen/carbon balance like, *H. lugdunensis*, *T. aquatica*, *T. tuberculata*, *A. crassa* FP 48, FP 154 and *M. aquatica* ; iv) finally those fungi that produce their antimicrobial activities specifically in plant derivatives such as pharmamedia or corn steep liquor, (*T. setigerum*, *T. elegans* and *V. elodeae* FP 164). The observation of these activities in these non-overlapping circumstances could be related to the presence of specific precursors of the active substance(s) in these media, as it was reported for the synthesis of penicillin G by *Penicillium chrysogenum* (Mead & Stack, 1948).

The production of the antimicrobial activity was neither associated with the growth level of the fungi on each specific substrate, nor with the ability to synthesize degrading enzymes such as cellulases or proteases (data not shown). However, these optimum conditions for antibiotic production could be hypothetically related to the degree of specificity of fungi for colonizing a given substrate, or for determining their role during the ecological succession of fungi in the decomposition of the plant substrates.

This work has shown that aquatic hyphomycetes are a potential source of uncharacterized bioactive compounds. To obtain a maximum information on the metabolic potential of these fungi, several media with different nitrogen/carbon balance should be applied, rather than different media with a low nitrogen/carbon balance, as it has been traditionally used (Poch & Gloer, 1992; Harrigan *et al.*, 1995). Other physical variables not

Source : MNHN, Paris

considered in this work, such as temperature or pH, might also influence the production of these still uncharacterized compounds.

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Source : MNHN, Paris